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MARINE BIOTOXINS: LABORATORY CULTURE AND MOLECULAR STRUCTURE

Annual Report

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Dinoflagellates, Gambierdiscus toxicus, were collected at different sites on the island of O'ahu. Cultures of a number of clones have been started and are being maintained. Ciguatoxin is being extracted from the viscera and flesh of yellowtail amberjack, Seriola dumerilii, which originated in the Northwest Hawaiian Islands and was shown to be toxic by the Hokama stick test.

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FOREWORD

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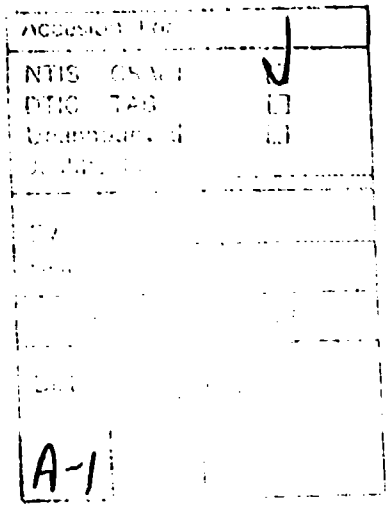

I. INTRODUCTION

A. The Problem

Marine biotoxins are among the most potent naturally occurring toxins known. Their physiological actions are diverse, as are their molecular structures, not all of which have been fully determined. Some of these toxins constitute a hazard to human health and safety. It is therefore imperative to elucidate their structures and to make pure toxins available for the study of pharmacological properties and mechanisms of action.

In this study we are principally concerned with two toxins that are associated with the human fish intoxication known as ciguatera, ciguatoxin and maitotoxin, both of unknown structure, and with palytoxin, the structure of which is known but unique and whose mechanism of action is not well understood. Each of the three toxins presents a different set of problems.

1. Ciguatoxin

It is present in ciguateric carnivorous fishes in concentrations ranging from 1 to 10 ppb. Its molecular structure is only partly known. It has been produced as a pure crystalline entity from toxic fish extracts but not from the toxic dinoflagellate Gambierdiscus toxicus, which is associated with ciguatoxic coral reefs.

2. Maitotoxin

In contrast to ciguatoxin, which is soluble in organic solvents, maitotoxin is water-soluble. It was first described from the gut of herbivorous toxic fishes, where it occurs in low concentration. It has been produced in a number of laboratories from cultured G. toxicus. Its molecular

structure is partially known.

3. Palytoxin

This toxin, whose structure has been known for several years, has so far been isolated from toxic zoanthid corals, Palythoa sp. Preliminary evidence suggests that the producer of this toxin is not the coral, but an epiphyte, possibly a bacterium.

B. Previous Work

1. Ciguatoxin

There is no convincing literature report that would indicate that anyone has yet succeeded in producing ciguatoxin from cultures of G. toxicus. Miller and coworkers[1] have cultured G. toxicus from a Caribbean collection. The nature of the toxin is difficult to assess as these workers report an LD_{50} of 4.95 mg/kg of the lipid extract after four chromatographic purification steps. This corresponds to but 0.01% of the lethality of pure ciguatoxin. Durant-Clement[2] has cultured G. toxicus from a Gambier islands strain and reports toxic extracts, water-soluble maitotoxin-like and lipid-soluble ciguatoxin-like, in ratios varying from 9:1 to 7.5:2.5.

The bulk of the work on the molecular structure of ciguatoxin, which remains to be fully elucidated, was performed by Tachibana.[3]

2. Maitotoxin

Maitotoxin has been produced from G. toxicus cultures in a number of laboratories. Yasumoto and coworkers[4] have had G. toxicus from the Gambier islands in culture since soon after the organisms was described.[5] They have

been producing maitotoxin consistently and have begun mass spectral and NMR studies to delineate its structure.

3. Palytoxin

The molecular structure of palytoxin is known,[6] but the suggestion[7] that the toxin is biosynthesized by an epiphyte of the zoanthid coral has not been realized.

C. Purpose of Present Work

The purposes of this work are the successful culture of G. toxicus; production of ciguatoxin and maitotoxin; structural elucidation of both toxins; and production of palytoxin in laboratory culture.

D. Method of Approach

1. G. toxicus culture

The methodology is known.[4] What is not known, however, are the factors, genetic and/or environmental, which govern production of maitotoxin and/or ciguatoxin. The approach must be empirical. We will collect G. toxicus from as many sites as possible and experiment with a variety of culture parameters.

2. The Molecular Structures of Maitotoxin and Ciguatoxin

Once the G. toxicus cultures are productive, they presumably will furnish maitotoxin. This toxin can be purified and its structure elucidated by well-known procedures and techniques.

Until such time, when the G. toxicus cultures produce ciguatoxin, we will continue to procure toxic carniverous fishes and purify ciguatoxin for

structural elucidation. Because of the extremely low concentration of toxin our goal will be the production of a crystalline derivative for structure determination by x-ray diffraction techniques.

3. Palytoxin Production from Cultures

We will commence this phase of the research once the toxin production from G. toxicus cultures has been achieved.

II. RESULTS TO-DATE

Because of initial administrative delays in activating this contract and on-going construction and equipping of a new culture laboratory, the following results do not represent an entire year's effort.

A. Laboratory Construction

Construction of the algal culture laboratory remains incomplete. The contractor has not yet completed construction of culture incubation rooms (controlled temperature rooms); until the refrigeration equipment in these rooms is operational, we cannot begin any extensive cultivation of G. toxicus as the heat produced by banks of fluorescent lights would rapidly kill the algae.

Additionally, construction of the "clean" room to be used for production of axenic algal isolates was completed only by September 20; hoods and other equipment have now been installed. We can now use this room for sterile culture manipulations.

2. Major equipment acquisition

a. Equipment detailed in contract proposal which has been acquired and is in operational condition:

Fermenters, 5 and 30 L working volume

Computer control system for above

Chiller for 30 L fermenter

Controlled rate freezer

Cryogenic container

Backup air compressor

Inverted Microscope

Backup generator

Low temperature freezer

Controlled environment shaker

Media Preparation equipment

Centrifuge rotor

b. Equipment detailed in contract proposal which has not yet been acquired:

Tissue homogenizer

Refrigerated recirculator for 5 L fermenter

Alarm system

c. Additional equipment, not listed in proposal, which we hope to be able to purchase with savings realized in "equipment purchased":

Microplate reader (to perform ELISA assays for ciguatoxin)

Mechanical shaker

It is worthy of note that the backup generator which was acquired for this research saved our organisms during an island-wide power outage on

Saturday, September 10. In sharp contrast, Professor Y. Hokama's laboratory lacks an emergency generator. The disastrous results are described in the article below from the September 17 issue of the Honolulu Advertiser.

A-6 Saturday, September 17, 1988 The Honolulu Advertiser

Power outage sets back research on fish toxin

By Vickie Ong
Advertiser Staff Writer

One billion mouse cells being cultured by a University of Hawaii researcher for a test to detect ciguatera toxin in fish were pronounced dead yesterday — victims of last Saturday's widespread power outage.

"I just checked and (my research associates) said the cells are all dead," said Dr. Yoshitugi Hokama, professor of pathology at the UH's School of Medicine.

Hokama has developed a simple stick test that uses small bamboo sticks coated with Liquid Paper and the mouse antibodies, which react to any ciguatera toxin in fish.

He had 100 milliliters of the culture — a little less than a half cup — in little vials in a computer-controlled incubator at the UH Biomedical Building. Last week's blackout interrupted power momentarily to the incubator, upsetting the temperature and carbon dioxide mix in the chamber for 24 hours.

"These cell cultures are shocked. The question is whether they will recover from this shock," Hokama said. He paused to check with his staff on how the cells were doing and came back with the bad news.

The loss of the cells, plus the labor involved in preparing the culture, will cost about $5,000 and about two months of work.

Fortunately, he said he has other batches of cells kept in "suspended animation" in the freezer and "we can go back and resurrect those cells." But it will take several weeks before they adapt to the culture and begin multiplying, Hokama said.

Hokama uses rapidly reproducing mouse cancer cells that are fused with normal, antibody-producing cells to make hybrid cells used in his tests.

Ciguatera poisoning results from the consumption of certain reef fish, including kole (surgeon fish), roi (groupers), kahala (amberjacks) and papio and ulua (jack). Symptoms include diarrhea, nausea, a tingling sensation or numbness of the mouth and hands, a reversed sensation of hot and cold, and erratic heart beat.

B. Establishment of G. toxicus cultures.

1. Dinoflagellate sources

G. toxicus culture samples have been requested from Drs. Sherwood Hall (USFDA), R. Bagnis (French Polynesia), and Jeffrey Bomber (SIU, Carbondale).

but none have so far been received.

Collections on the island of O'ahu have been made on four field trips to Kahala twice and once each to the reef runway at Hickam Air Force Base and to Black Point. Table I lists the results of these collections.

Table I. Algal Collections and Dinoflagellate Epiphytes

ALGAL SPECIES	SITE	DEPTH	SUBSTRATE	DINOFLAGELLATE
Spyridia filamentosa	Kahala Beach	1.0m	sand/robble	Positive
	Black Point	1.0m	sand/robble	Positive
Laurencia sp.	Kahala Beach	1.0m	Rock	Negative
Halimeda opontia	Kahala Beach	0.5m	sand	Negative
Acanthophora sp.	Reef runway	0.5m	rock	Positive
Padina sp.	Kahala Beach	1.0m	rock	Positive
	Reef runway	0.8m	sediment	Negative
Sargassum sp.	Kahala Beach	1.0m	rock	Negative
Turbinaria ornata	Kahala Beach	1.0m	rock	Negative
Dictyota dichotoma	Kahala Beach	0.5m	sand	Negative

Collections on the island of Tarawa failed to survive the return journey. A total of 105 clones are in the process of being established and cultured. Twenty-one of these clones have been tentatively identified as Ostreopsis siamensis and 70 as Gambierdiscus toxicus. A further fourteen clones have yet to be identified.

Initial isolation attempted using medium K resulted in poor dinoflagellate growth. The reason for this may have been related to a change

in the pH of the medium from 8.15 to 7.4 over a period of seven days. Greater success has been achieved using Provasoli's ES medium, with nine strains of Ostreopsis siamens being transferred to 16mm diameter borosilicate tubes as a preliminary step prior to large-scale culture being attempted.

All experiments conducted to date have involved the isolation and culture of single dinoflagellates collected form the surface of benthic macroalgae. As previously reported by Shimizu et al.[8] Gambierdiscus toxicus has been found to be associated with the red alga Spyridia filamentosa, whereas the principal dinoflagellate associated with Acanthophora sp. was Ostreopsis siamensis. Upon arrival of calibrated water counting chambers, it will be possible to quantify the relationship between different dinoflagellate species and their algal substrates. If all progresses to plan, enough cells should be obtained within the next month to allow for scaling up of the culture of individual clones, particularly Gambierdiscus toxicus.

2. Culturing Methodology

All glassware used in isolation procedures was soaked in 10% hydrochloric acid for 48 hours and washed thoroughly with distilled water prior to use. Aged filtered (1.4 um) seawater was used in the preparation of all culture media. The methods used to isolate epiphytic dinoflagellates from macroalgae are essentially as described by Carlson.[9] Briefly, samples of algae were sealed within plastic bags with a minimum of disturbance. Specimens of each alga collected were preserved in 5% formalin in seawater for later identification and for assessment of the substrate specificity of the dinoflagellate species.

On return to the laboratory, the bags containing the algae were shaken

vigorously and the cell suspension obtained examined microscopically for the presence of dinoflagellates. Single cells were removed from the suspension using sterile micropipettes and transferred to 0.5 mL of sterile culture media dispensed into each well of a 24 well tissue culutre plate (Costar Pty. Ltd.). Two different culture media have been used for the initial isolation and cloning of dinoflagellates. These media are the ES-enriched natural seawater of Provasoli[10] and medium K of Keller and Guillalrd.[11] Both culture media were supplemented with antibiotics (chloramphenicol, 5mg/L and rifampicin, 1mg/L). The culture plates were incubated at 25° C with 34 watt cool white fluorescent tubes providing constant illumination (33 uE.m^2 .sec^{-1}).

Tentative identification of the dinoflagellates isolated has been made according to Steidinger[12] and Carlson.[9]

C. Molecular Structure of Ciguatoxin

The island of Tarawa, Republic of Kiribati, continues to experience high incidence of ciguatera. The Chief Medical Officer has reported 142 documented cases of ciguatera poisioning during the period January 1 to September 7, 1988. There is currently no air freight service between Tarawa and Hawaii so that no toxic fish can be obtained from that source. A new carrier may start service in 1989.

We have therefore processed sixty specimens of yellowtail amberjack (Seriola dumerilii) from Midway Island. A large catch, many specimens of which tested positive in the Hokama stick test, was donated to the University. Between March and September, 1988 we processed 55.6 kg of viscera and 348 kg of flesh with an estimated expected yield of 65 µg of toxin. We are accumulating the toxin at the gel filtration stage.

Approximately 900 kg of amberjack remain to be processed. Additionally, approximately 150 kg of Moray eels which tested positive have been received.

Our procedure is essentially as described previously,[13] with these modifications. Initial freeze-drying of the thawed fish greatly accelerates the extraction with acetone. Substituting ethyl acetate for ether in the toxin separation proved to be highly efficient.

D. Palytoxin Production by Bacterial Culture

Our initial search for a qualified postdoctoral researcher was unsuccessful.

III. CONCLUSIONS

A new laboratory for algal culture is almost completed. A number of G. toxicus clones have been established, they are growing. We expect to begin maitotoxin production during the coming months.

Ciguatoxin production from fish is proceeding and crude toxin is being accumulated.

Search for a qualified person to undertake bacterial culture will be resumed.

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